

**Generation of Fluorescent Recombinant Listeriolysin O Toxin for Analysis of Interactions with Host Proteins**

A Senior Honors Thesis

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## A - Introduction

### A.1 – *L. monocytogenes* and Listeriosis

*Listeria monocytogenes* is a food-borne Gram-positive pathogen that is the causative agent of listeriosis (2). Listeriosis is a life-threatening illness, which is primarily a threat to immunocompromised individuals and pregnant women. *L. monocytogenes* causes various symptoms ranging from a mild chill and gastroenteritis to more severe illnesses and diseases such as premature birth or miscarriage, and meningitis in newborns. In immunocompromised adults, the bacteria can cause septicemia or meningitis (4).

### A.2 – Listeriolysin O (LLO) as the major virulence factor

The major virulence factor of *L. monocytogenes* is listeriolysin O (LLO). *L. monocytogenes* is a facultative, intracellular pathogen (7) that can invade phagocytic and non-phagocytic cells (1). In non-phagocytic cells, *L. monocytogenes* actively invades the non-phagocytic cell via interactions between the internalin surface proteins (Internalin A and Internalin B) and the host membrane receptors E-cadherin and HGF factor (5). LLO has been shown to play crucial roles in bacterial intracellular survival by mediating the disruption of the host vacuolar membranes after uptake of the bacteria, hence releasing the bacteria into the cytosol (2).

### A.3 – LLO and the CDC family of Toxins

LLO is a member of the Cholesterol-Dependent Cytolysin (CDC) family of pore-forming toxins (2). The CDC proteins share 40-70 % amino acid sequence similarity, which allows for the assumption that they share similar three-dimensional structures and mechanisms of action (6). The crystal structures of the CDC proteins Perfringolysin O (PFO) and

Intermedilysin (ILY) have been determined (7). These two proteins consist of four domains similar in structure, which are most likely representative of all of the family members. PFO and LLO present 42 % amino acid sequence similarity and 68 % similarity in Domain 4 (7). Therefore, PFO is used to model the structure of LLO (Figure 1).

#### *A.4 – Pore Formation*

The exact mechanism for CDC binding and pore-formation is not completely understood; however, it is thought that CDCs bind to the cell membrane via domain 4 at the tryptophan rich undecapeptide sequence and loops L1 to L3. The protein then oligomerizes in a ring structure called a pre-pore complex before penetrating the membrane to form a pore. The mechanism of membrane perforation has been extensively studied and it is thought that large scale conformational remodeling in domain 3 unfolds the  $\alpha$ -helices to span the membrane and create a pore. The pore is composed of 35 to 50 monomeric proteins and has a diameter of about 300 Angstroms (2).

#### *A.5 – Project Objective*

The goal of my project was to obtain recombinant LLO proteins which, when fluorescently labeled, can be used to determine the spatiotemporal dynamics of LLO with the host cells. Through the experimental approaches of my project, we will generate a recombinant toxin which can be fluorescently labeled so that the mechanism of action of the domains of LLO can be studied. In order to achieve this goal, two major steps had to be taken:

- 1) Construct, purify, and fluorescently label recombinant toxin.

- 2) Analyze, by hemolytic assay, the activity of the fluorescently labeled recombinant toxin and determine if the labeled toxin binds to host membranes by fluorescence microscopy

This project is important because it will lay the groundwork for understanding the interactions between CDCs and the host cell, by using LLO as a model CDC. The results will lead to a better understanding of diseases generated by Gram-positive bacteria. Also our results will allow us to refine the design of antitoxins to fight infectious diseases.

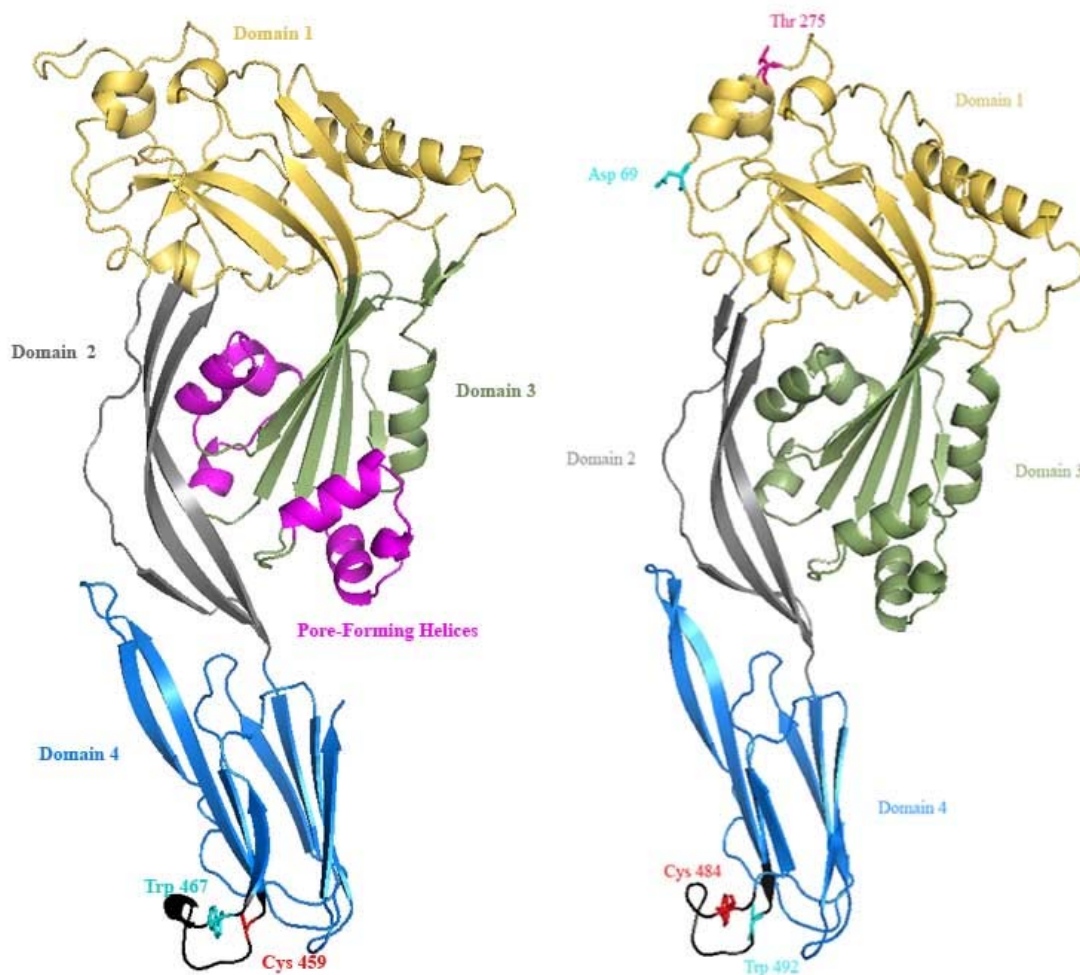


Figure 1. Determined crystal structure of PFO is shown on the left and the modeled structure of LLO is shown on the right. The four domains are distinguished by different colors and have been properly labeled. The undeca-peptide sequence in domain 4 has been shown in black and the pore forming helices have been labeled in the PFO structure.

## B - Materials and Methods

### B.1 - Site-Directed Mutagenesis

#### Construction of the cysteine-free LLO<sub>C484A</sub>-6xHis

- Mutation of the Cys to an Ala in D4

The first step in creating protein mutants began through manipulation of plasmid pet29b with an encoding region for LLO and resistance to the antibiotic kanamycin. This plasmid contained a C-terminal 6xHis tag in the LLO gene, which was later used for protein purification. Initially, the plasmid was purified using a DNA miniprep kit (Qiagen).

#### Mutagenesis

The initial step was to replace the only cysteine codon at amino acid 484 within the LLO sequence with an alanine codon. This was attained through performing a site-directed mutagenesis with Stratagene's Quikchange site-directed mutagenesis kit. To obtain the desired mutation, two complementary oligonucleotides were designed and ordered (Integrated DNA Technologies, Inc.) that contained the desired base pair mutation flanked by unmodified nucleotide sequence (Table 1). Primers were designed so that the mutation was in the middle of the primer sequence. Primers needed at least one G or C at each end, and a total of 25-45 bp to obtain a melting temperature (T<sub>m</sub>) of 78°C or higher. The primers needed a T<sub>m</sub> of 78°C so they could bind to DNA during the annealing and elongation phases of the Polymerase Chain Reaction (PCR). The primers were reconstituted in water to give final concentrations of 125 ng/μl. This was to facilitate adding primers to the PCR reaction mixture.

AA mutation (original->mutant)	Original LLO plasmid DNA sequence (Top Strand)	Forward Primer (T <sub>m</sub> )	Reverse Primer (T <sub>m</sub> )
<b>AA #484</b> <b>(Cys-&gt;Ala)</b>	5' -ATTAATGTTTACGCTAAAG AATG <b>C</b> ACTGGTTTAGCTTGGAAT GG-3'	5' -ATTAATGTTTACGCTAAAG AA <b>GCG</b> ACTGGTTTAGCTTGGA ATGG-3' (T <sub>m</sub> = 78.5°C)	5' -CCATTCCAAGCTAAACCA GT <b>CGC</b> TTCTTTAGCGTAAACAT TAAT-3' (T <sub>m</sub> = 78.5°C)

<b>AA #27</b> (Ala->Cys)	5'-GAAGGAGATATACATATGA AGGATG <b>C</b> ATCTGCATTCAATAA AGAAAATTCAATTTTCATCC-3'	5'-GAAGGAGATATACATATGA AGGAT <b>TGCT</b> CTGCATTCAATAA AGAAAATTCAATTTTCATCC-3' (T <sub>m</sub> = 78.2°C)	5'-GGATGAAATTGAATTTTCT TTATTGAATGCAGAG <b>CA</b> ATCC TTCATATGTATATCTCCTTC- 3' (T <sub>m</sub> = 78.2°C)
<b>AA #69</b> (Asp->Cys)	5'-GAAATCGATAAGTATATAC AAGGATTG <b>G</b> ATTACAATAAAAA CAATGTATTAGTATACCACG-3'	5'-GAAATCGATAAGTATATAC AAGGATTG <b>TGT</b> TACAATAAAAA CAATGTATTAGTATACCACG- 3' (T <sub>m</sub> = 78.6°C)	5'- CGTGGTATACTAATACATTG TTTTTATTGT <b>ACA</b> CAATCCTT G TATATACTTATCGATTTC- 3' (T <sub>m</sub> = 78.6°C)
<b>AA #274</b> (Val->Cys)	5'-CCAGATTTTTTCGGCAAAGC TGTTAAAGAGCAGTTGCAA GCG- 3'	5'-CCAGATTTTTTCGGCAAAGC T <b>TG</b> C AAAGAGCAGTTGCAA GCG-3' (T <sub>m</sub> = 79°C)	5'-CGCTTGCAACTGCTCTTT AGT <b>GCA</b> AGCTTTGCCGAAAAAT CTGG-3' (T <sub>m</sub> = 79°C)
<b>AA #275</b> (Thr->Cys)	5'-CCAGATTTTTTCGGCAAAGC TGTT <b>ACT</b> AAAGAGCAGTTGCAA GCGC-3'	5'-CCAGATTTTTTCGGCAAAGC TGTT <b>TGT</b> AAAGAGCAGTTGCAA GCGC-3' (T <sub>m</sub> = 81.4°C)	5'-CGCTTGCAACTGCTCTTT <b>ACA</b> AACAGCTTTGCCGAAAAAT CTGG-3' (T <sub>m</sub> = 81.4°C)

TABLE 1. Primers used to mutate the LLO gene. The codon highlighted in blue represents the original codon which was mutated and the codons highlighted in red represent the mutagenic region of the oligonucleotide primer.

The plasmid was then subjected to PCR using the complementary primers. The reaction mixture contained: 125 ng (1 µl) of each primer, 1 µl of dNTP mix, 1 µl of 10x reaction buffer, 50 ng of pet29b plasmid and water. The total amount of water added was dependent on the volume needed to add 50 ng of plasmid. As a positive control, the plasmid DNA and oligonucleotides were replaced with the PCR control plasmid pWhitescript and the mutagenic primers provided by Stratagene. 1 µl of *PfuUltra* DNA polymerase (2.5 U/µl) was used in each mixture. Each tube was then subjected to PCR in a thermocycler for 16 cycles which served to melt the DNA, anneal the primers, and finally elongate the DNA with the desired mutations (Table 2). After PCR, the reaction mixture was placed on ice for 2 minutes to cool.

Segment	Cycles	Temperature	Time
1 (denaturation)	1	95°C	30 sec
2 (denaturation)	16 (18 - control)	95°C	30 seconds
(annealing)		55°C	1 min
(elongation)		68°C	7 minutes (5 min – control)
3 (holding temp)	1	4°C	∞

Table 2. PCR cycling protocol for site directed mutagenesis

### Digestion and Transformation

The parental DNA in the PCR mixture was then digested with 1 µl of DpnI (10 U/µl) restriction enzyme (New England Biolabs). DpnI digested the parental DNA which had been methylated by the XL1-Blue *E. coli*. Sample was incubated for 1 hour at 37°C to allow the DpnI enzyme to function efficiently. The plasmid DNA was then transformed into competent XL1-Blue *E. coli* cells by adding 5 µl of DNA to 100 µl of competent XL1-Blue cells. Transformed cells were plated on a LB kan plates to select for the bacteria which had taken up the plasmid DNA. Plates were incubated overnight at 37°C.

### Purification and Sequencing of the Mutated Plasmid

The following day, five colonies were picked from the LB-kan plate. The colonies were each inoculated into 5 ml of LB kan broth and allowed to grow overnight. The following morning, the plasmids were purified from the bacterial cultures. The bacterial pellets formed during centrifugation were purified using a plasmid purification kit (Qiagen).

The optical densities at 260 nm and 280 nm ( $OD_{260}/OD_{280}$ ) were then measured to determine the concentration and purity of the DNA. The gene encoding for LLO was then sequenced to confirm that the mutagenesis was successful. This was performed by using T7P and T7T oligonucleotide primers which attach to the plasmid before and after the LLO gene. The sequencing results were aligned using BioEdit DNA alignment software. After determining that the mutation of a Cysteine to an Alanine was successful, the cysteine-free LLO gene (LLO<sub>C484A</sub>-6xHis) became the template sequence for all future reconstructions of the toxin.



### Construction of LLO<sub>C484A</sub>–6xHis

#### ▪ Insertion of Cys in D1

The LLO<sub>C484A</sub>–6xHis encoding gene was subjected to an additional mutation using the same site-directed mutagenesis protocol as described above. Three mutants corresponding to different site of mutation were constructed. All primers were reconstituted in a volume of water which gave a final concentration of 125 ng/μl. The amino acids that were selected were determined to likely be of lesser importance in the mechanism of action of LLO based upon their location within the 3D structure (Figure 1). Also amino acids were selected around the edges of the protein structure because this would allow for measuring FRET between different monomer of toxin. Four point mutations in domain 1 were attempted in order to increase the odds that at least one recombinant would maintain activity after purification and fluorescent labeling. Conversions of alanine 27, aspartate 69, valine 274, and threonine 275 to cysteine were attempted. Mutagenic primers were used to introduce these mutations into domain 1 of LLO which are presented in Table 1.

Recombinant toxins (KDa)	Mutations
LLO C484A-6xHis (57)	C484 A (TGC→GCG)
LLO C484A/A27C-6xHis (57)	C484A (TGC→GCG) A27C (GCA→TGC)
LLO C484A/D69C-6xHis (57)	C484A (TGC→GCG) D69C (GAT→TGT)
LLO C484A/T275C-6xHis (57)	C484A (TGC→GCG) T275C (AC→TGC)
Table 3. Recombinant toxins created via site-directed mutagenesis.	

## *B.2 - Protein Purification*

### Protein Synthesis

In order to produce and purify the LLO recombinant toxins, plasmids encoding for each recombinant toxin were transformed into BL21 (DE3) *E. coli* cells. 50 μl of BL21 (DE3) cells

were grown overnight on LB kanamycin plates after transformation with 5  $\mu$ l of each plasmid. The following day, a colony was picked and inoculated into 10 ml LB kanamycin broth overnight. Each overnight culture was then added to 500 ml LB kan. The culture was incubated with shaking at 37°C until it reached an OD<sub>600</sub> of 0.6. This was to ensure that the cells were in the exponential growth phase before LLO protein synthesis was induced. LLO production was induced by adding 500  $\mu$ l (1  $\mu$ l/ml) of the chemical Isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG), which binds to the LacI product which represses the lac operator that is the promoter for the LLO gene. After 5 hours at 25°C, the culture was divided centrifuged at 3,000 rpm for 45 min at 4°C (Beckman J2-MC, JA14 rotor). After centrifugation, the bacterial pellet was frozen at -80°C.

The following day, the bacterial cultures were resuspended in 1.5 ml of 50 mM phosphate buffer at pH 8 (1 M NaCl, 5 mM  $\beta$ -Mercaptoethanol, 0.75 mg/ml DNase I, and 50  $\mu$ l/(g of *E. coli*) of protease inhibitor). The *E. coli* suspension was then lysed in a Mixer Mill 200 (Retsch) at -20°C and at a frequency of 30 sec<sup>-1</sup> for 9 min. The lysed cells were centrifuged at 14,000 rpm for 30 min at 4°C (Beckman Microfuge 22R).

#### Affinity Chromatography

The clear lysate was then applied to a nickel-nitrilotriacetic acid (Ni-NTA) matrix by rotating for two hours at 4°C. Protease inhibitors were added to the mixture at a concentration of 50  $\mu$ l/(g of *E. coli*) to prevent protein degradation. The matrix was bound to the LLO protein via the 6xHis tag located at the C-terminus of the toxin. After application to the matrix, the sample was thoroughly washed with three different buffers before protein elution. 8 total washes were performed with a 50 mM phosphate/acetate buffer at pH 8 (1M NaCl and 5 mM  $\beta$ -mercaptoethanol). Six washes were then performed with a 20 mM phosphate/acetate buffer at

pH 6 (1M NaCl, 20mM imidazole, 5 mM  $\beta$ -mercaptoethanol, 0.1 % Tween 20, and 10 % glycerol). After these washes the OD<sub>280</sub> of the solution was measured; if the OD<sub>280</sub> was less than 0.01, the final two washes were performed. These final washes consisted of the previous buffer without tween or glycerol. After each wash, the matrix was centrifuged at 1,500 rpm for 5 minutes at 4°C in a Beckman Allegra 6R centrifuge to pellet the matrix and aspirate the wash buffer.

### Elution

Once the wash steps were complete, the matrix which contained the protein was transferred to a plastic column at 4°C for elution. This was performed by adding 1.5 ml of elution buffer to the column after rinsing the original wash tube with the buffer. The elution was performed with a 50 mM phosphate/ acetate buffer at pH 6 containing 1 M NaCl, 500 mM imidazole, and 5 mM  $\beta$ -mercaptoethanol. An additional 1.5 ml was used to rinse the original wash tube a second time, which was immediately applied to the elution column. The flow through was collected in 0.5 ml aliquots.

The elution profile was analyzed via DC protein assay (Bio-Rad) to determine which fraction contained a high concentration of protein. 5  $\mu$ l of each aliquot was added to a separate well in a 96 well plate. A protein standard was prepared by adding 10  $\mu$ l of BSA at a concentration of 1.75 mg/ml and diluting it in elution buffer via serial dilution. This protein served as a standard for comparison with the protein suspensions from the elution. 25  $\mu$ l of reagent A was added to each well (Bio-Rad). Reagent A is a copper solution that reacts with the protein. After reagent A, 200  $\mu$ l of reagent B (Bio-Rad) was added to each well. Reagent B is a Folin reagent that is reduced in the presence of the copper-treated protein. After 15 minutes, the absorbance was analyzed at OD<sub>750</sub> to determine which fraction contained the eluted protein.

### Dialysis

The concentrated toxins were then dialyzed to remove unwanted small molecules. Dialysis was performed by injecting the toxin into a Slide-A-Lyzer dialysis cassette (Thermo Scientific). The toxin was dialyzed overnight at 4°C with stirring in a 50 mM phosphate/acetate buffer at pH 6 containing 0.5 M NaCl, 1 mM EDTA, and 170 µM TCEP. The following day the cassette was placed in fresh dialysis buffer for an additional 4 hours. This buffer was similar to the first dialysis buffer except it did not contain TCEP. After dialysis, the OD<sub>280</sub> of the protein was measured to determine the concentration. 5 µl of the protein was collected and diluted in 95 µl of elution buffer to test the concentration of the protein without using a significant size of the sample. Dialysis buffer diluted 20x was used as a blank.

### Analysis

After each major step of the protein purification process, aliquots were collected and analyzed in an SDS-PAGE gel. Also, western blots were performed to ensure that the LLO protein was not degraded. To perform a western blot the LLO protein was run on an SDS-PAGE gel and then the protein was transferred to a Polyvinylidene Fluoride (PVDF) membrane. After blotting, non-fat dry milk was applied to the membrane to saturate the PVDF with proteins. A rabbit anti-His-tag polyclonal antibody was used as the primary antibody in detection. The secondary antibody was horseradish-peroxidase-linked secondary antibody (anti-rabbit IgG) which produces luminescence in proportion to the amount of the primary antibody, and antigen product on the membrane.

### *B.3 -- Fluorescent Coupling*

After the proteins had been purified, they were fluorescently labeled with the molecular probe Alexa Fluor 488 (Invitrogen) C5 maleimide. To optimize labeling, the recombinant

proteins were diluted into a labeling buffer to obtain a concentration of 2.8 mg/ml. The labeling buffer used for diluting the proteins was similar to the final dialysis buffer of protein purification and consisted of 50 mM phosphate/ acetate, pH 6.8, 0.5 M NaCl, 1 mM EDTA. After dilution, the fluorescent probe was added to the protein suspension. To prepare the dye, 140  $\mu$ l of labeling buffer was added to 1 mg of Alexa Fluor 488. 25  $\mu$ l (0.2 mg) of dye was then added to each mixture. Each labeling reaction was prepared so that a final volume of 0.5 ml of sample was obtained. The samples were incubated with stirring at 4°C for a total of 5 hours to allow the fluorescent probe to couple to the protein at the cysteine residue. The reaction was protected from light by wrapping the tubes with aluminum foil.

After the 5 hours of incubation, the labeled toxin was separated from the uncoupled dye via gel filtration chromatography (Sephadex G25). The column used was a Sephadex PD-10 desalting column (GE). Before application of the sample to the column for filtration, the column was prepared by applying 2.5 ml of labeling buffer to equilibrate the column. The sample was then applied to the column. After the sample had completely entered the column, 2 ml of additional labeling buffer was added for a total volume of 2.5 ml. The flow through was collected in aliquots of 1 ml, 1 ml, 0.5 ml, and 0.5 ml for the remaining aliquots. An additional 3.5 ml of buffer was added to allow the sample to flow through the column completely. The aliquots that were collected were analyzed by DC protein assay to determine which fractions contained the labeled protein. As a negative control the fluorescent dye was added to the LLO<sub>C484A</sub> toxin.

After filtration was complete and the proteins had been collected, the OD<sub>280</sub> and OD<sub>494</sub> were measured so that the concentration of protein and dye in each sample could be calculated. The following equations were used to determine the molar concentration of the protein and the

dye to protein ratio. When necessary, the samples were diluted so that the OD<sub>280</sub> and OD<sub>494</sub> readings were below 1.0.

$$\frac{[Abs_{280} - (Abs_{494} * 0.11)] * dilution}{75,750} = [M] \text{ concentration of protein.}$$

$$\frac{Abs_{494} * dilution}{71,000 * protein[M]} = \text{dye/protein ratio.}$$

#### B.4 -- Hemolytic Assay

After fluorescently labeling the toxins, the hemolytic ability of the proteins was tested. First, a suspension of sheep red blood cells 0.2 % (SRBCs) was obtained. 750 µl of stock 10 % SRBCs (Lampire) were washed with 30 ml cold Phosphate Buffered Saline at pH 7.4 (PBS) and the suspension was centrifuged at 1500 rpm for 15 min at 4°C in a Beckman Coulter Allegra 6R centrifuge. The supernatant was aspirated without removing any of the SRBCs. SRBCs were resuspended in 30 ml fresh PBS and the process was repeated for a total of three washes. After the final wash, the SRBCs were resuspended in 30 ml PBS to obtain a SRBC suspension of 0.2 % in PBS.

During the preparation of the SRBC solution, a round-bottom 96-well plate was prepared. For each protein to be tested, a row was filled with 20 µl PBS (except the first column). Additionally, Triton 1 % diluted in PBS and PBS served as positive and negative controls, respectively. 20 µl of each LLO protein to be tested was added to column 1 and 2 of each row at a concentrations of 0.0026 mg/ml for LLO<sub>C484A/T275C</sub>-6xHis, 0.0032 mg/ml for LLO<sub>C484A/D69C</sub>-6xHis, 0.0044 mg/ml for LLO<sub>C484A/A27C</sub>-6xHis, 0.45 mg/ml for LLO<sub>W492A</sub>-6xHis, and 0.01 mg/ml for LLO<sub>WT</sub>-6xHis (Table 4). A serial dilution of each toxin was performed starting with 20 µl from column 2. This dilution was performed for each row containing an LLO toxin. 200 µl of

0.2 % SRBCs in PBS at pH 7.4 was then added to each sample. All of steps were performed on ice to prevent protein degradation.

Samples were allowed to incubate on ice for 5 min after SRBCs had been added. Then the samples were incubated for 30 minutes at 37°C. LLO<sub>WT</sub> was used as a control for the normal amount of lysis of SRBCs by LLO. After incubation and cell lysis, the plate was centrifuged in an Allegra 6R centrifuge to pellet the intact red blood cells. The supernatant containing hemoglobin from the lysed red blood cells was then transferred to a flat-bottom 96 well plates so that the absorbance of the hemoglobin solution could be measured at 450 nm. The results were analyzed to determine the concentration of each recombinant protein that causes 50 % SRBC lysis.

Toxin	Concentration
LLO WT-6xHis	0.01 mg/ml
LLO W492A-6xHis	0.45 mg/ml
LLO C484A/A27C-6xHis	0.0044 mg/ml
LLO C484A/D69C-6xHis	0.0032 mg/ml
LLO C484A/T275C-6xHis	0.0026 mg/ml
<b>Table 4. Recombinant toxins that were tested in the hemolytic assay along with the initial concentration for each serial dilution.</b>	

## B.5 -- Fluorescence Microscopy

### Vero Cells Cultured

After the toxins had been fluorescently labeled and had been determined to maintain their pore-forming capabilities, they were tested for their ability to bind to mammalian cell membranes. The cells used were African green monkey kidney epithelial (Vero) cells. First, Vero cells were cultured in a 75 cm<sup>2</sup> flask in ATCC complete growth medium. This was Minimal Essential Medium (MEM - Eagle) with 2 mM L-glutamine and Earle's BSS adjusted to

contain 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, and 1.0 mM sodium pyruvate, 90 %; fetal bovine serum, 10 %. Cells were subsequently incubated at 37°C in an atmosphere composed of 95 % air and 5 % CO<sub>2</sub> for 2 days.

After 2 days, the culture medium was removed from the cells and discarded. The cells were rinsed with 1 ml 0.25 % (w/v) Trypsin- 0.53 mM EDTA to remove all traces of serum that contained trypsin inhibitor. The trypsin-EDTA solution was then removed. 1 ml of trypsin-EDTA was subsequently added to the flask to detach the cells. Cells were observed under a microscope until cell layer was dispersed (5-10 min). 9 ml of fresh growth culture medium (MEM) was immediately added to the flask to stop the trypsin reaction. Cells were resuspended by gently pipetting. A sample of the cells was removed to determine the cell density. Once the density of the culture was known, cells were diluted and seeded at 10<sup>5</sup> cell/35mm glass bottom culture dish. 2 ml was added to each dish. Cells in 35 mm dishes were placed in the incubator at 37°C.

#### Addition of LLO<sub>C484/D69C</sub> 6xHis-A488 to Vero Cells

The following day, the cells were ready to be labeled with LLO-A488. To prepare the cultures for fluorescence imaging, the cells were washed with 3x1 ml of cold PBS. LLO<sub>C484/D69C</sub> 6xHis-A488 was then diluted to a final volume of 1 ml in cold PBS and added to the Vero cells at concentrations of 1 nM, 2 nM, and 4 nM for each culture. One 35 mm dish of Vero cells had only PBS added to serve as a negative control. Each dish was incubated on ice for 5 min in the dark. Unbound toxin was removed by washing with 3x1 ml of cold PBS. 500 µl of Para-formaldehyde 4 % was added to each dish to fix the cells. Cells were washed again with 3x1 ml cold PBS. This protocol was followed with 2 additional dishes in which 2 nM of LLO<sub>D69C</sub> -



A488 was added to the cells, the only difference being that the cells were also incubated at 37°C for 1 min and 5 min after the unbound toxin was removed.

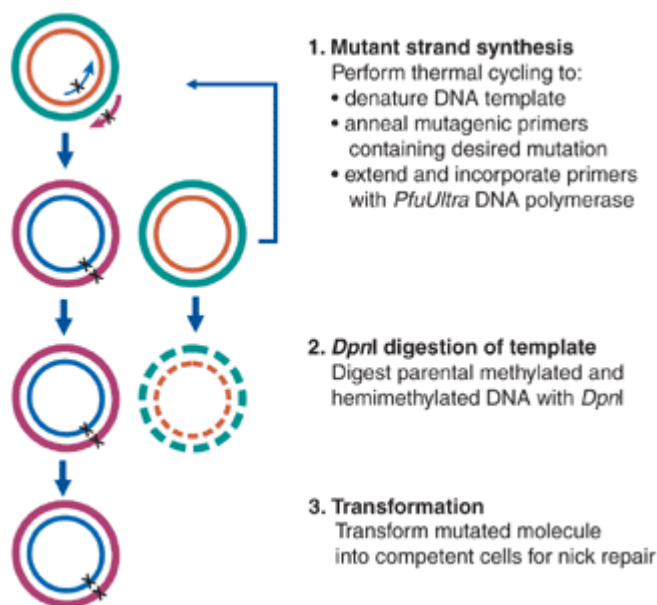
### Images Acquired

After the toxin had been applied to the Vero cells, cells were viewed using a Zeiss Observer D1 microscope under 100x objective. Fluorescence and phase contrast images were acquired using MetaMorph computer software. The average fluorescence intensity of the cells were calculated for each concentration of protein.

## **C - Results and Discussion**

### *C.1 -- Site Directed Mutagenesis*

The QuikChange site directed mutagenesis kit (Stratagene) is a reliable and simple way to generate point mutations in plasmids. The QuikChange protocol uses mutagenic primers, which overlap onto complementary DNA strands within a plasmid after denaturation. The aim is to target a specific amino acid within the primer sequence to be mutated (Figure 3). After attachment of the primers, *PfuUltra* DNA polymerase replicates the plasmid via PCR (Fig. 2). Over several cycles an abundance of mutated DNA that is not methylated is synthesized by *PfuUltra*. The parental DNA, which had been methylated by the XL1-Blue *E. coli*, was successfully digested using DpnI, which left only the unmethylated DNA synthesized during PCR.



**Figure 2. QuikChange Site Directed Mutagenesis Protocol**

The OD<sub>260</sub> and OD<sub>280</sub> of the plasmids which were purified from the XL1-Blue cells were determined to be pure however lower than the desired concentration for sequencing of 50 ng/μl (Table 5). The valine 274 to cysteine mutation was unsuccessful and no clones were obtained after transformation. The protocol was continued with only the other three successful mutations. The concentrations of the purified clones which were submitted to sequencing are shown in Table 5. In spite of the low concentration of plasmid, all ratios were roughly 1.8 and the Plant Microbe Genomics Facility at Ohio State sequenced the plasmid successfully.

Purified Clones	Concentration	OD <sub>260</sub> /OD <sub>280</sub>
LLOC484A-6xHis - #2	18 ng/μl	1.9
LLOC484A-6xHis - #4	16.5 ng/μl	1.9
LLOC484A/A27C-6xHis - #3	21 ng/μl	1.83
LLOC484A/A27C-6xHis - #5	23.5 ng/μl	1.88
LLOC484A/D69C-6xHis - #1	80 ng/μl	1.85
LLOC484A/D69C-6xHis - #2	23.5 ng/μl	1.68
LLOC484A/T275C-6xHis - #3	58.5 ng/μl	1.77

Table 5. List of purified plasmid DNA mutants that were used for further analysis along with their concentration and purity at the time of sequencing

The results from DNA sequencing revealed successful point mutations for all plasmids which were submitted to the Plant Microbe Genomics Facility for sequencing. The cysteine-free mutant contained a point mutation of a Cys-to-Ala at the 484<sup>th</sup> amino acid, as desired. The other mutants, which were further constructions of the Cysfree toxin, revealed their desired point mutations. Due to the success of the mutations of the genes encoding for LLO, we continued to the next stage of the experiment with confidence that the desired recombinant proteins could be purified.

### C.2 -- Protein Purification

The protocol required that the plasmids with mutated genes encoding for LLO be transformed into BL21 (DE3) *E. coli*. This strain of *E. coli* exhibits a high level of protein synthesis and permitted a high level of expression of LLO after induction with IPTG. Figure 3 shows the high level of LLO expression that resulted from induction with IPTG. The induction of LLO in BL21 (DE3) *E. coli* was successful in all instances.

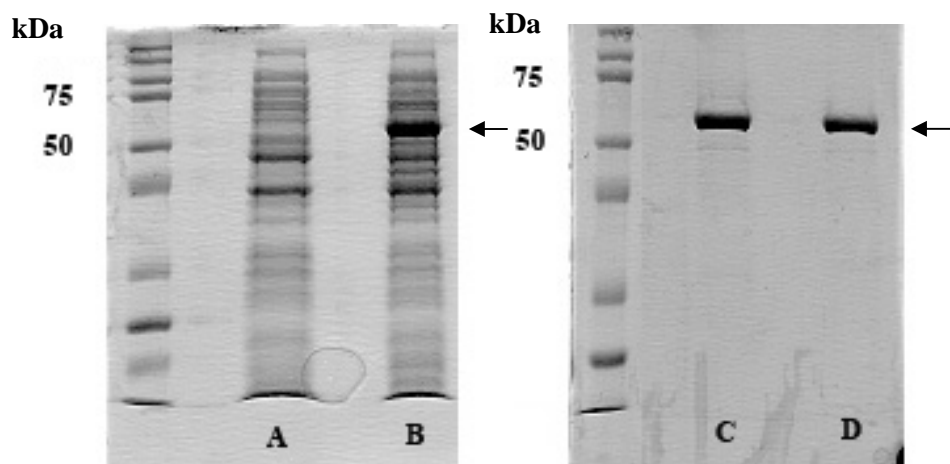


Figure 3. BL21(DE3) lysates before (A) and after (B) induction of LLO<sub>A27C</sub>. Arrows show LLO<sub>A27C</sub> (C) and LLO<sub>A27C</sub>-A488 (D). Arrows show the LLO<sub>A27C</sub> toxin.

After induction of LLO, lysis of *E. coli* cells became a problem in the purification protocol. Initially, the cells were sonicated on ice. This protocol proved to be inefficient due to two problems. The sonicator was not capable of efficiently lysing the cells for lower outputs. When the output was increased, the cells began to overheat and the LLO toxins denatured. Correcting these two problems was impossible, so a new strategy was developed. The new strategy involved lysing cells in an MM200 (Retsch). This strategy was effective. The cells did not overheat using this method and were well lysed.

Purification of the protein via affinity chromatography was successful. This protocol involved binding of the Ni-NTA matrix to the 6xHis tag at the C-terminus of the recombinant toxins. This is the result of interactions of the  $\text{Ni}^{2+}$  ions with the 6xHis tag. The 6xHis tag and the Ni-NTA matrix were successfully bound by incubation and rotation. The high yield of protein after elution and analysis on an SDS-PAGE gel indicated that the protein and matrix were successfully bound (Figure 3).

After the wash steps, the proteins were successfully eluted. The protein was present at a high concentration in elution fraction #2 based on results of the DC protein assay. SDS-PAGE gel and Western Blot results revealed that the LLO toxin with a 6xHis tag was successfully purified. The concentrations based upon the  $\text{OD}_{280}$  readings for each recombinant toxin were 8.5 mg/ml for LLO<sub>A27C</sub>, 3.46 mg/ml for LLO<sub>D69C</sub>, and 9.88 mg/ml for LLO<sub>T275C</sub> (Table 6).

Recombinant Toxins	Concentration
LLO <sub>A27C</sub>	8.5 mg/ml
LLO <sub>D69C</sub>	3.46 mg/ml
LLO <sub>T275C</sub>	9.88 mg/ml

**Table 6. Concentration of each recombinant toxin after purification via affinity chromatography.**

### *C.3 -- Fluorescent Labeling*

The successfully purified LLO toxins were then covalently conjugated to the Molecular Probe Alexa-Fluor 488 C5 Maleimide (Invitrogen). Alexa Fluor 488 is known to be the best green-fluorescent reactive dye available and it has a high level of photostability. Alexa Fluor 488 has an excitation/emission maxima of 495/519 nm. The maleimide binds to the unique cysteine residue in the LLO toxin. The cysteine residue originally located in domain 4 (Cys 484) was removed from the undecapeptide sequence in domain 4 of the protein, because oxidation of this cysteine inhibits the pore forming ability of the toxin (3).

The elution profiles of the fractions from Sephadex gel filtration chromatography show that the fluorescent labeling was effective (Figure 4). The protocol revealed a successfully labeled protein that was eluted in the 5<sup>th</sup> fraction sample. The elution also clearly showed that the unbound dye was eluted after the labeled protein. The toxin in this fraction had a dye/ protein ratio of 0.88 (Figure 5). The negative control, LLO<sub>C484A</sub>, revealed that there was unbound dye in elution 5. LLO<sub>C484A</sub> had a dye/ protein ratio of 0.3, which was likely a result of electrostatic reactions between the negatively charged Alexa Fluor 488 dye and the positively charged amino acids within the protein sequence. Because of these results, it was clear that the protein was labeled; however, perhaps to a lesser degree than what was being calculated. The results of fluorescent coupling were dye/ protein ratios of 0.92 for LLO<sub>A27C</sub>, 0.85 for LLO<sub>D69C</sub>, and 0.97 for LLO<sub>T275C</sub>. The concentration of protein and dye in the other elution fractions of gel filtration were not determined, because the protein had very clearly eluted into the 5<sup>th</sup> fraction.

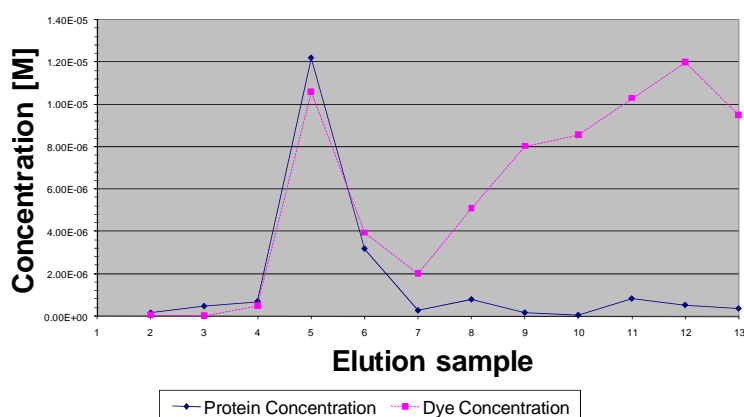


Figure 4. Elution profiles of LLO<sub>WT</sub>-A488 and free dye. Fraction 5 is collected.

#### C.4 -- Hemolytic Assay

A hemolytic assay was performed as an indicator of the pore-forming ability of the toxins by measuring the lysis of sheep red blood cells (SRBCs). LLO binds to red blood cells and lyses them by oligomerizing and forming pores. The results of the hemolytic assay demonstrated that the recombinant, labeled toxins maintained hemolytic ability. The LLO<sub>A27C</sub> maintained complete hemolytic ability and exhibited 50 % hemolysis at a concentration of 0.7 nM, which actually proved better than the LLO<sub>WT</sub>, which had 50 % hemolysis at 1 nM. The other two recombinant toxins, LLO<sub>D69C</sub>, and LLO<sub>T275C</sub> maintained approximately 50 % hemolysis when compared to the LLO<sub>WT</sub>. Their concentrations at 50 % lysis were 1.8 nM (Figure 5). Because of these results, all 3 fluorescently labeled recombinant toxins were determined to be capable of forming pores.

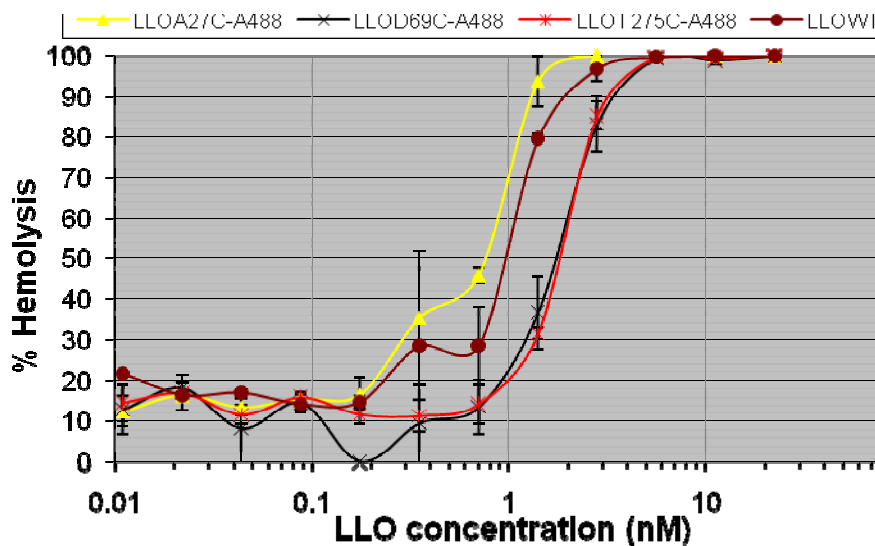


Figure 5. The hemolytic curves were determined by the level of hemoglobin released from a 0.2 % suspension of SRBCs measured at 450 nm.

### C.5 -- Fluorescence Imaging

Toxins were added to the cell at 4°C, a temperature that impedes all internalization of the membrane, therefore if the toxin interacts with the cell, they do so by association with the plasma membrane. LLO<sub>D69C</sub>-A488 proved effective at interacting with Vero cells. The toxin was clearly visible during acquisition of the cell images. The cells that were incubated on ice for 5 min with the toxin demonstrated increased levels of fluorescence intensity when the concentration of protein added was increased (Figure 7). The cell cultures that contained 2 nM of toxin and were incubated at 37°C so that the toxin had the opportunity to perforate the cells showed that LLO was perforating the membrane and invading the cells. The cells incubated for 1 minute did not display internalization, however, the cells that were incubated for 5 minutes were. This revealed that the cell was being lysed at a point between 1 min and 5 min at a concentration of 2 nM of toxin. Images that demonstrate this have been provided (Figure 6). 6 images were acquired under phase contrast and with a GFP filter so that the average fluorescence intensity of the cells at each concentration of toxin could be determined. The cells that contained 0 nM, 1

nM, 2 nM, and 4 nM of toxin displayed an average fluorescence intensity of 26.8/pixel, 72.9/pixel, 89.6/pixel, and 172.3/pixel, respectively (Figure 7). These results demonstrated that the fluorescent recombinant toxins bind to mammalian membranes and form pores in the host membrane. Therefore, the goal of this project was achieved. Three fluorescent recombinant LLO toxins were obtained that could bind to host cell membranes, form pores, and be efficiently visualized by fluorescence microscopy.

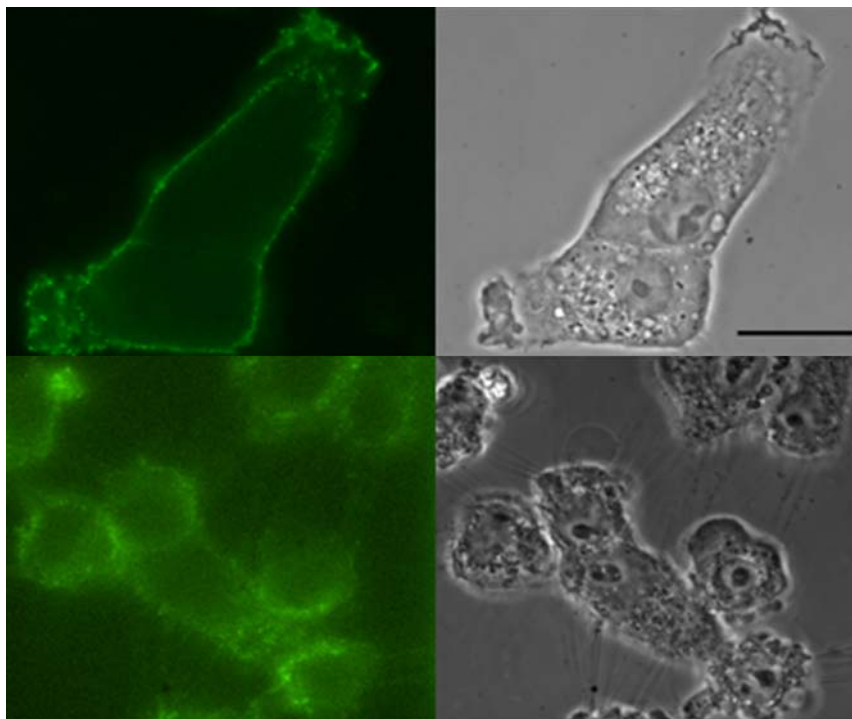
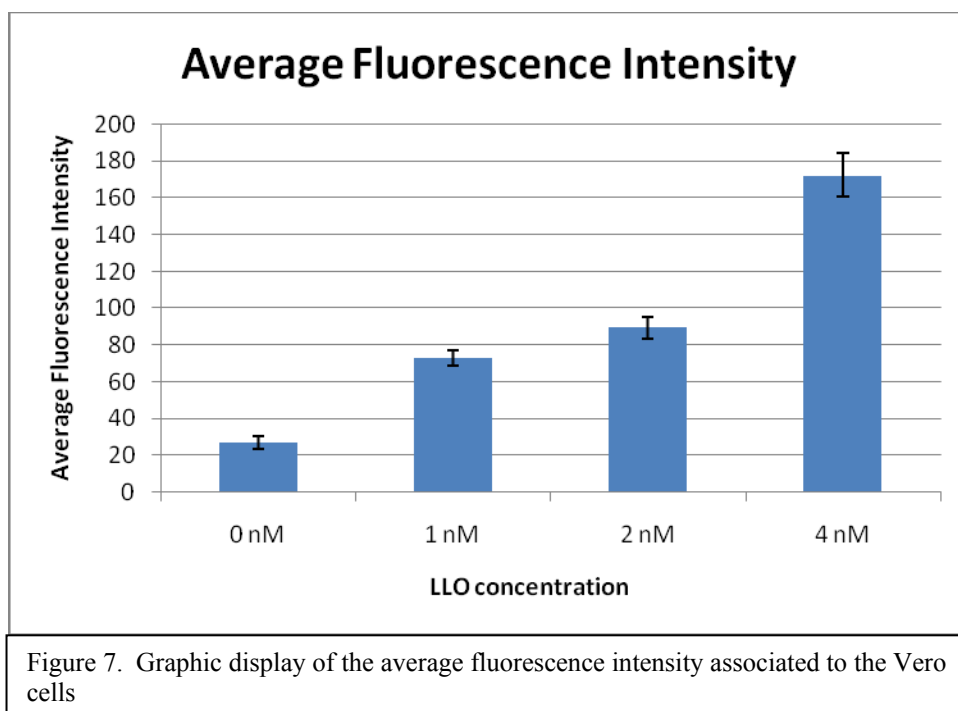


Figure 6. Top left image shows a Vero cell that was labeled with 4 nM of LLO<sub>D69C</sub>-A488 and visualized with a GFP filter. Top right shows the same cell under phase contrast. Bottom left and right show cells that were labeled with 2 nM of LLO<sub>D69C</sub>-A488 that were incubated for 5 min at 37°C under GFP and phase contrast respectively. Scale Bar = 20 mm.





## D - Future Research

A fluorescent LLO which maintains pore-forming capability has been obtained for the first time. These fluorescently labeled LLO toxins can be used to study the interactions of LLO with host cells in order to determine the dynamics of toxin clustering and interaction with membrane lipid rafts. Also fluorescent toxin will provide a powerful tool to determine the internalization pathway and intracellular trafficking of the toxin. Through the knowledge gained from better understanding the interactions of CDCs such as LLO with host cells we can begin to refine the design of antitoxins to fight infectious diseases.

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